[CANCER RESEARCH 52, 5954–5962, November 1, 1992]

A Murine Model for Antibody-directed Targeting of Vascular Endothelial Cells in Solid Tumors[1]

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ABSTRACT

An attractive approach to the therapy of solid tumors would be to target cytotoxic agents or coagulants to the vasculature of the tumor rather than to the tumor cells themselves. This strategy has 3 advantages: (*a*) it should be applicable to many types of solid tumors because all require a blood supply for survival and growth; (*b*) the target endothelial cells are directly accessible through the blood and are normal cells, making the outgrowth of resistant mutants unlikely; and (*c*) there is an in-built amplification mechanism because thousands of tumor cells are reliant on each capillary for nutrients and oxygen. Despite its theoretical attractions, the approach of tumor vascular targeting has not been testable because antibodies that recognize tumor vascular endothelial cell antigens with adequate specificity are currently not available. In this study, we developed a model system in which to investigate the antibody-directed targeting of vascular endothelial cells in solid tumors in mice. A neuroblastoma transfected with the mouse interferon-γ gene, *C1300(Muγ)*, was grown in antibiotic-treated BALB/c nude mice. The interferon-γ secreted by the tumor induces the expression of major histocompatability complex Class II antigens on the tumor vascular endothelium. Class II antigens are absent from the vasculature of normal tissues, although they are present on B-lymphocytes, cells of monocyte/macrophage lineage, and some epithelial cells. Anti-Class II antibody administered i.v. strongly stains the tumor vasculature, whereas an antitumor antibody directed against a major histocompatability complex Class I antigen of the tumor allograft produces classical perivascular tumor cell staining. This model should enable the theoretical superiority of tumor vascular targeting over conventional tumor cell targeting to be tested.

INTRODUCTION

In contrast with their efficacy in lymphomas (1, 2), monoclonal antibodies and immunoconjugates have proved relatively ineffective in the treatment of the major carcinomas (3, 4). The principal reason for this is that solid tumors are rather impermeable to antibody-sized molecules: specific uptake values of less than 0.001% injected dose/g of tumor are not uncommon in human studies (5, 6). Furthermore, antibodies that enter the tumor mass do not distribute evenly for 3 reasons. (*a*) The dense packing of tumor cells and fibrous tumor stroma present a formidable physical barrier to macromolecular transport and, combined with the absence of lymphatic drainage, create an elevated interstitial pressure in the tumor core that reduces extravasation and fluid convection (7, 8). (*b*) The distribution of blood vessels in most tumors is disorganized and heterogeneous, so that some tumor cells are separated from extravasating antibody by large diffusion distances (8). (*c*) All of the antibody entering the tumor may become adsorbed in perivascular regions by the first tumor cells encountered, leaving none to reach tumor cells at more distant sites (7, 9–11).

A solution to the problem of poor penetration of antibodies into solid tumors is to attack the endothelial cells lining the blood vessels in the tumor. This approach offers 3 advantages over direct targeting of tumor cells. (*a*) The target cells are directly accessible to i.v. administered therapeutic agents, permitting rapid localization of a high percentage of the injected dose (9). (*b*) Since each capillary provides oxygen and nutrients for thousands of cells in its surrounding "cord" of tumor, even limited damage to the tumor vasculature could produce an avalanche of tumor cell death (12, 13). (*c*) The outgrowth of mutant endothelial cells lacking the target antigen is unlikely because they are normal cells.

For tumor vascular targeting to succeed, antibodies are required that recognize tumor endothelial cells but not those in normal tissues. Although several antibodies have been raised (14–17), none has shown the required degree of specificity. However, numerous differences between tumor blood vessels and normal ones have been documented (reviewed in Refs. 12 and 18–20) that suggest that such antigenic differences exist. Tumors elaborate angiogenic factors (21) and cytokines (22, 23), which alter the behavior and phenotype of local endothelial cells. Vascular endothelial cells in tumors incorporate [^{3}H]-thymidine at a rate 30-fold greater than those in miscellaneous normal tissues (24), suggesting that proliferation-linked determinants could serve as markers for tumor vascular endothelial cells. Tumor angiogenesis requires detachment of endothelial cells from their underlying basement membrane before migration (21), so it is possible that antibodies to integrin extracellular matrix receptors may be selective for tumor endothelial cells because integrins are sequestered on the basal surfaces of normal quiescent endothelia (25, 26).

In this report we describe a murine model for antibody-directed targeting of tumor vascular endothelial cells. We show that an IFN[3]-γ-producing tumor growing in antibiotic-treated BALB/c *nu/nu* mice induces the expression of MHC Class II antigens on the tumor vascular endothelium. MHC Class II is absent from the vasculature of normal tissues, although it is present on B-lymphocytes, cells of monocyte/macrophage lineage, and some epithelia. Anti-Class II antibody administered i.v. localizes rapidly and strongly to the tumor vasculature, whereas an antitumor antibody, directed against a Class I antigen of the tumor allograft, produces classical perivascular tumor cell staining. This model should thus enable the theoretical superiority of tumor vascular targeting over conventional tumor cell targeting to be tested.

MATERIALS AND METHODS

Animals. BALB/c *nu/nu* mice were purchased from Simonsen (Gilroy, CA). All animals were maintained in microisolation units on

Received 4/8/92; accepted 8/21/92.

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[1] This work was in part supported by NIH Grant 1RO1CA 54168–02.

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[3] The abbreviations used are: IFN, interferon; BSA, bovine serum albumin; CD, cluster determinant; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; MEM, modified Eagle's medium; MHC, major histocompatibility complex; rIFN-γ, recombinant murine interferon-γ; NK, natural killer; PBS-BSA-N_3, phosphate-buffered saline containing 0.2% (w:v) bovine serum albumin and 0.2% (w:v) NaN_3; PBS-T, phosphate-buffered saline containing 0.05% (v/v) Tween 20 (Sigma).

sterilized food and water. Where indicated, tetracycline-HCl (Vedeo, St. Joseph, MO) was added to drinking water to a final concentration of 1.1 mg/ml (27). The strain carries the H-2^d haplotype.

Cells and Culture Conditions. All cell lines used in this study were cultured in MEM supplemented with 10% (v/v) fetal calf serum, 2.4 mM L-glutamine, 200 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 90% air/10% CO_2. The C1300 neuroblastoma line was established from a spontaneous tumor that arose in an A/Jax mouse in 1940 (28). The C1300(Muγ)12 line, hereafter referred to as C1300 (Muγ), was derived by transfection of C1300 cells with the murine IFN-γ gene using the IFN-γ expression retrovirus pSVX (MuγδA_s) (29, 30), and was cultured in MEM as above containing 1 mg/ml G418 (Geneticin; Sigma). Both lines carry the MHC haplotype H-$2K^k$, I-A^k, I-E^k, D^d. C1300 and C1300(Muγ) cells were grown in regular tissue culture flasks or, when large quantities were required for *in vivo* experiments, in cell factories (Baxter, Grand Prairie, TX). Cells from s.c. tumors were recovered for *in vitro* analysis by gentle mincing in MEM. After tumor cells had adhered overnight, the monolayers were washed twice with MEM to remove nonadherent contaminant host cells. Tumor conditioned media were prepared by seeding C1300 and C1300(Muγ) cells at 25% of confluent density and culturing them for 4 days. Conditioned media were dialyzed for 16 h against MEM without FCS to remove G418, filtered through a 0.22-μM membrane, and stored at 4°C for no more than 1 week before assay. Aliquots of anti-IFN-γ antibodies (see "Monoclonal Antibodies") sufficient to neutralize 200 IU of murine IFN-γ/ml of conditioned medium were added to some samples 24 h before assay. The SVEC-10 murine endothelial cell line, hereafter referred to as SVEC, was kindly provided by Dr. M. Edidin, Department of Biology, The Johns Hopkins University, Baltimore, MD, and was derived by immortalization of lymph node endothelial cells from a C3H (H-2^k) mouse with SV40 (31). For some experiments, SVEC cells were cultured for 72 h with 100 IU/ml rIFN-γ (a generous gift from Dr. F. Balkwill, Imperial Cancer Research Fund, London, England) or tumor-conditioned medium. In addition, 200 IU/ml anti-IFN-γ antibody were added to some flasks at the beginning of the 72-h culture period.

Monoclonal Antibodies. The M5/114.15.2 (hereafter referred to as M5/114) and 11–4.1 hybridomas were purchased from the American Type Culture Collection (Rockville, MD) and were grown in MEM-10% FCS. The antibodies were purified from culture supernatant by precipitation in 50% ammonium sulfate and affinity chromatography on Protein A. The rat IgG2b antibody, M5/114, detects an Ia specificity on I-A^b, I-A^q, I-A^d I-E^d, and I-E^k molecules (32). Thus, the antibody recognizes I-E^k molecules on SVEC (H-2^k) cells and I-A^d and I-E^d, hereafter referred to collectively as Ia^d, on cells from BALB/c *nu/nu* mice (H-2^d haplotype). The anti-Ia^d reactivity of M5/114 was confirmed in this study by FACS analyses with the Ia^d-expressing B-lymphoma line, A20/25 (33). The mouse IgG2a antibody 11–4.1 recognizes H-$2K^k$ but not H-$2K^d$ molecules (34), and so binds to H-$2K^k$ on C1300 and C1300(Muγ) cells but is unreactive with MHC antigens from BALB/c *nu/nu* mice. Isotype-matched control antibodies of irrelevant specificity were CAMPATH-2 (rat IgG2b, anti-human CD7) (35) and WT-1 (mouse IgG2a, anti-human CD7) (36). Purified preparations of CAMPATH-2 and WT-1 were generous gifts from Dr. G. Hale (Department of Pathology, Cambridge University, Cambridge, England) and Dr. W. Tax (Sint Radboudzeikenhuis, Nijmegen, The Netherlands), respectively. Rat anti-mouse endothelial cell antibody MECA-20 (14) was donated as a concentrated culture supernatant by Dr. A. Duijvestijn (University of Limburg, The Netherlands) and used at a dilution of 1:200 for indirect immunoperoxidase staining. Rat antibodies against mouse macrophages (anti-Mac-1, M1/70) and mouse CD3 (KT 31.1) were generously provided by Dr. P. Beverley (Imperial Cancer Research Fund, London, England) and the rat IgM antibody CZ-1, which reacts with B-cells, CD8+ T-cells, and NK cells but is specific for NK cells in SCID mice (37), was kindly provided by Dr. R. M. Welsh (University of Massachusetts Medical Center, Worcester, MA). Hamster anti-mouse IFN-γ antibody 1222–00 (38), used for specific neutralization of IFN-γ *in vitro*, was purchased from Genzyme (Boston, MA). Anti-mouse IFN-γ antibodies, XMG1.2 and R46A2, used in IFN-γ ELISAs, were kindly provided by Dr. N. Street (University of Texas Southwestern Medical Center, Dallas, TX). Purified 11–4.1, WT-1, and XMG1.2 antibodies were biotinylated by incubation with a 12.5-fold molar excess of *N*-hydroxysuccinimidobiotin amidocaproate (Sigma) for 1 h at room temperature followed by dialysis against 2 changes of PBS.

ELISA for Murine IFN-γ. Sandwich ELISAs for murine IFN-γ were carried out as described previously (39). In brief, the wells of flexible polyvinyl chloride microtiter plates (Dynatech, Alexandria, VA) were coated with 50 μl/well of a 2 μg/ml solution of capture anti-IFN-γ antibody, R46A2, in PBS for 2 h at room temperature. Nonspecific protein binding sites were blocked with 20% FCS in PBS for 15 min at 37°C. The plates were washed 3 times in PBS-T, and 25 μl/well control and experimental samples in MEM-10% FCS were added. After incubating for 1 h at 37°C, the wells were washed as before and 50 μl/well of a 1 μg/ml solution of biotinylated anti-IFN-γ antibody XMG1.2 in PBS-T containing 1% BSA were added. After incubation for 30 min at 37°C, the wells were washed as before and incubated with 75 μl of a 1:2000 dilution of horseradish peroxidase-conjugated streptavidin (DAKO) for 1 h at room temperature. After thorough washing in PBS-T, the wells were incubated for 30 min with 100 μl/well of a 1 mg/ml solution of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) in citrate/phosphate butter containing 0.003% (v:v) H_2O_2. Reaction product was measured as A_{405}-A_{490}. IFN-γ levels in experimental samples were calculated by reference to a recombinant murine IFN-γ standard solution in MEM-10% FCS.

Indirect Immunofluorescence. SVEC, C1300, and C1300(Muγ) cells were prepared for FACS analyses as described previously (22). All manipulations were carried out at room temperature. In brief, 50 μl of a cell suspension at 2–3 × 10^6 cells/ml in PBS-BSA-N_3 were added to the wells of round-bottomed 96-well microtiter plates (Falcon 3910). Optimal dilutions of rat or mouse antibodies were distributed in 50-μl volumes, and the plates sealed. After 15 min, the cells were washed 4 times by centrifuging the plates at 800 × *g* for 30 s, removing the supernatants, and resuspending the cells in 150 μl/well PBS-BSA-N_3. Fluorescein isothiocyanate-conjugated rabbit antibodies against rat or mouse IgG (ICN, High Wycombe, England), diluted 1:20 in PBS-BSA-N_3, were distributed in 50-μl volumes into the appropriate wells. The cells were incubated for a further 15 min and washed as before. Cell-associated fluorescence was measured on a FACScan (Becton-Dickenson, Fullerton, CA). Data were analyzed using the CONSORT 30 program.

Preparation of Tissues and Immunohistochemistry. For the establishment of solid tumors, a total of 2 × 10^7 C1300 or C1300(Muγ) cells, or a mixture of the two, in 200 μl MEM-30% FCS was injected s.c. into the right anterior flank of BALB/c *nu/nu* mice. Tumor diameters were measured at regular intervals and the animals were euthanized after 16 days (rapidly growing tumors) or 20 days (slowly growing tumors). Tumors and normal tissues were excised immediately and snap-frozen over liquid nitrogen. Normal tissues were also harvested from non-tumor-bearing animals. Antibody localization experiments were performed in animals bearing 1 cm s.c. tumors induced by injection of C1300 and C1300(Muγ) in the ratio 7:3. One hundred μg of unconjugated M5/114 or CAMPATH-2 antibodies or 100 μg biotinylated 11–4.1 or WT-1 antibodies in 100 μl PBS were injected i.v. At various times thereafter, the animals were euthanized and their circulation was flushed with PBS for 5 min before removal and freezing of tumors and normal tissues as before. Eight-μM frozen sections were cut on a Tissuetek 2 cryostat (Baxter) and air-dried for 2 h at room temperature. Slides were stored at −20°C for up to 3 months before assay. Indirect immunoperoxidase staining for rat IgG was adapted from a method described previously (40). Briefly, sections were allowed to return to room temperature, air dried for 30 min, and fixed in acetone for 15 min. After rehydration in PBS for 5 min, sections were incubated in a humidified chamber for 45–60 min with primary antibodies, diluted optimally in PBS-0.2% BSA. After 2 washes in PBS, the sections were incubated for 30–45 min with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts, Carpinteria, CA) diluted 1:10 in PBS-0.2% BSA supplemented with 20% normal mouse serum (ICN) to block antibodies cross-reacting with mouse immunoglobulins. After a further 2 washes in PBS, the reaction product was developed using 0.5 mg/ml

3',3'-diaminobenzidine (Sigma) containing 0.01% (v/v) hydrogen peroxide for 8 min. The sections were counterstained with Mayer's hematoxylin (Sigma) for 15 s, dehydrated in absolute ethanol, cleared in xylene, and mounted with Accumount 60 medium (Baxter). Indirect immunoperoxidase staining with biotinylated mouse antibodies was carried out in the same manner, except that peroxidase-conjugated streptavidin-biotin complex, diluted 1:50 in PBS with no blocking serum, was used as the second layer.

RESULTS

Murine IFN-γ Levels in C1300(Muγ)-conditioned Medium. C1300(Muγ)-conditioned medium contained 50.2–63.5 IU/ml murine IFN-γ, in accordance with previous reports (30). By contrast, less than 5 IU/ml IFN-γ were detected in C1300-conditioned medium or C1300(Muγ)-conditioned medium to which an excess of neutralizing anti-IFN-γ antibody had been added 24 h before assay.

Induction of MHC Class II (I-E^k) on SVEC Cells by rIFN-γ in C1300(Muγ)-conditioned Medium. As shown in Fig. 1*a*, unstimulated SVEC cells did not express I-E^k. By contrast, a large majority of cells preincubated with rIFN-γ (Fig. 1*a*) or with C1300(Muγ)-conditioned medium (Fig. 1*b*) expressed significant levels of I-E^k, and this induction was almost completely blocked by anti-IFN-γ. Treatment of SVEC cells with rIFN-γ or C1300(Muγ)-conditioned medium did not cause nonspecific antibody binding since the isotype-matched control antibody did not bind to the cells. These results were confirmed by indirect immunoperoxidase staining of cytospin preparations (data not shown).

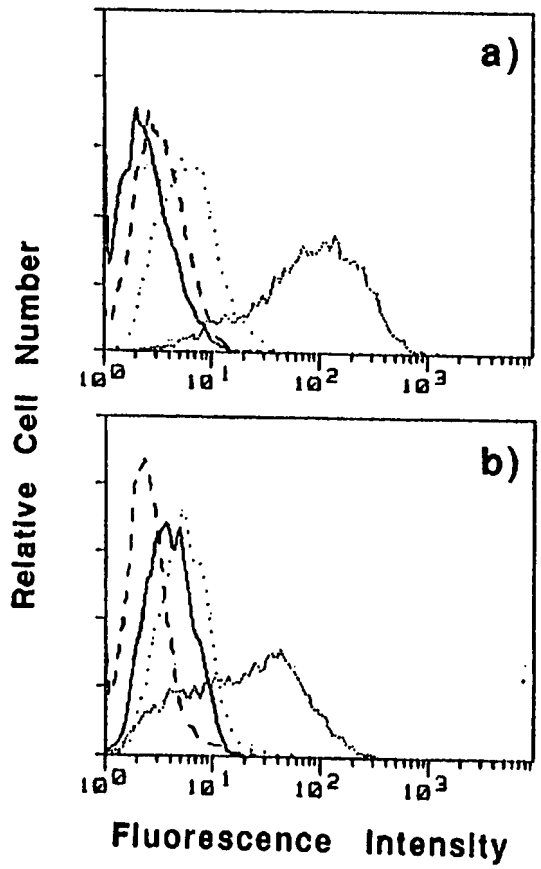

Fig. 1. Induction of I-E^k on SVEC cells by IFN-γ in C1300(Muγ)-conditioned medium. *a*, SVEC cells were cultured for 72 h in regular medium (—), rIFN-γ (. . . .), or rIFN-γ plus excess neutralizing anti-IFN-γ antibody (. . . .). Their expression of I-E^k was then measured by M5/114 antibody binding by indirect immunofluorescence using the FACS. Other cultures were treated with rIFN-γ and stained with an isotype-matched control antibody (- - - -). *b*, SVEC cells were cultured for 72 h in C1300-conditioned medium (—), C1300(Muγ)-conditioned medium (. . . .), or C1300(Muγ)-conditioned medium plus excess neutralizing anti-IFN-γ antibody (. . . .). Their expression of I-E^k was then measured as in *a*. Other cultures were treated with C1300(Muγ)-conditioned medium and stained with an isotype-matched control antibody (- - - -).

These findings suggested that vascular endothelial cells in tumors containing sufficient quantities of IFN-γ-secreting C1300(Muγ) cells should be induced to express high cell surface levels of MHC Class II molecules.

Expression of MHC Class I (H-2K^k) and Class II (I-E^k) by C1300 and C1300(Muγ) Cells. Since IFN-γ can induce MHC Class II antigen expression in diverse cell types (41–43) and since the M5/114 antibody cross-reacts with I-E^k, we determined whether the M5/114 antibody, intended for use to target tumor endothelial cells *in vivo*, would also (Fig. 2*a*) bind to the tumor cells themselves. As shown in Fig. 2*a*, C1300(Muγ) cells expressed I-E^k, but at levels 10–20-fold lower than those on SVEC cells stimulated with IFN-γ.

Similarly, C1300 cells expressed detectable but low levels of H-2K^k, whereas C1300(Muγ) cells displayed uniformly high levels, approximately 20-fold greater than on the parental line (Fig. 2*b*). This result was expected from the known autocrine Class I-inducing activity of IFN-γ and is in keeping with a previous report (30). Coculture of C1300(Muγ) cells and C1300 cells induced homogeneous expression of I-E^k and H-2K^k on both populations (Fig. 2). Induction of these antigens on C1300 cells appears to be caused by IFN-γ released into the culture medium by the C1300(Muγ) cells since the effect was neutralized by anti-IFN-γ antibodies (30).

Growth of C1300 and C1300 (Muγ) Tumors in Immunodeficient Mice and Induction of Ia^d on Tumor Vascular Endothelial Cells. We first attempted to grow s.c. C1300(Muγ) tumors in BALB/c *nu/nu* mice because the strain carries the MHC haplotype (H-2^d) with which the anti-MHC Class II antibody M5/114 reacts, and because they would not be expected to reject the tumors, as do syngeneic immunocompetent A/J animals (30). For unknown reasons, inocula composed entirely of C1300-(Muγ) cells failed to produce progressively growing tumors in BALB/c *nu/nu* mice. Conversely, pure C1300 inocula displayed 100% tumorigenicity but, as expected, did not contain Ia^d-positive endothelial cells. To identify a combination that would yield a high percentage of tumor takes and reliable growth kinetics, and cause Ia^d induction of a large majority of intratumoral endothelial cells, several ratios of C1300 and C1300-(Muγ) cells were inoculated into BALB/c *nu/nu* mice. As shown in Fig. 3, mixtures containing C1300 and C1300(Muγ) cells in the ratio 9:1 produced rapidly growing tumors but, when sections of the tumors were stained with anti-Ia^d antibody by the indirect immunoperoxidase technique, none of the endothelial cells in the tumor was found to be stained. Dropping the ratio of C1300:C1300(Muγ) to 8:2 gave rapidly growing tumors in which approximately 50% of blood vessels were Ia^d-positive. Dropping the ratio further to 7:3 or 5:5 produced tumors that grew quite rapidly and contained a large majority of Ia^d-positive vessels. Dropping the ratio still further to 3:7 produced tumors in no more than half of the animals, and those tumors that became palpable failed to grow beyond 6 mm in diameter. Histological analyses of the latter revealed no morphologically recognizable intact blood vessels and, hence, it was not possible to ascertain their level of Ia^d expression.

Of the 2 usable C1300:C1300(Muγ) ratios identified, 7:3 and 5:5, the ratio of 7:3 was adopted for the remainder of this study because the take rate was higher (100% *versus* 80%) and the

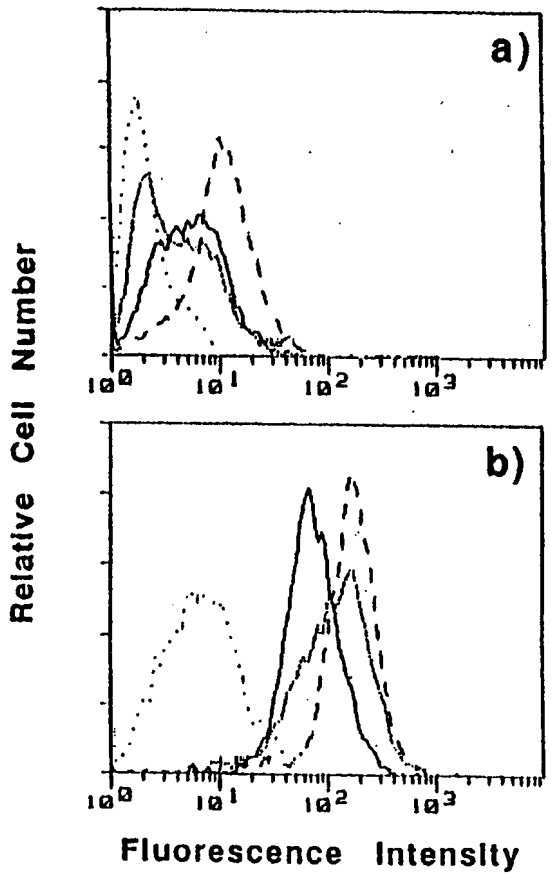

Fig. 2. Expression of I-E^k and H-2K^k by pure and mixed populations of C1300 and C1300(Muγ) cells. C1300 cells (. . . .), C1300(Muγ) cells (- - - -), a mixture of C1300 and C1300(Muγ) cells in the ratio 7:3 cocultured *in vitro* (. . . .), or cells recovered from a mixed s.c. tumor in a BALB/c *nu/nu* mouse (——) were stained with (*a*) anti-I-E^k antibody or (*b*) anti-H-2K^k antibody by indirect immunofluorescence using the FACS. No staining of any tumor cell population was seen with the isotype-matched control antibodies.

variability in tumor growth rate between individual animals was lower (data not shown).

Distribution of Ia^d in BALB/c Nude Mice. The distribution of M5/114 binding in tissues from tumor-bearing BALB/c *nu/nu* mice is shown in Table 1. In s.c. tumors, most or all vascular endothelial cells and numerous interstitial macrophages were stained (Fig. 4). In most organs, the binding of M5/114 reflected the classical distribution of MHC Class II antigens, being restricted to B-cells in lymphoid organs, resident macrophages in all tissues studied except brain, tissue-specific elements of the reticuloendothelial system, such as liver Kupffer cells and Langerhans cells of the skin, and to a minor subpopulation (5–8%) of bone marrow cells. In addition, staining was occasionally seen in some kidney tubules. When sections of small and large intestine from BALB/c *nu/nu* mice were examined, heavy labeling of both epithelial and endothelial cells was seen in both regions. The staining of *nu/nu* mouse intestine was found to be related to the microbiological status of the animals and is discussed below. Apart from in the gut, no staining of endothelial cells with M5/114 was seen in any tissues examined. The distribution of Ia^d antigens in normal tissues was not affected by the presence of the tumor because the staining pattern of M5/114 was identical in non-tumor-bearing mice.

Attenuation of Ia^d Expression on Intestinal Endothelium and Epithelium of Nude Mice by Administration of Antibiotics. In BALB/c *nu/nu* mice, most epithelial cells from all regions of the gut were intensely stained with anti-Ia^d antibody. In addition, some endothelial cells in both upper and lower bowel bound M5/114 antibody, particularly those associated with villi of the small intestine (Fig. 5*a*). When the animals were treated with p.o. tetracycline-HCl, a broad-spectrum antibiotic, for 1–3 weeks there was a progressive dimunition of Ia^d expression in the ileum and elsewhere in the gut, so that binding of M5/114 was in most sections restricted to the luminal membranes of a minority of epithelial cells (Fig. 5*b*). Light cytoplasmic staining of occasional endothelial cells was observed in some antibiotic-treated animals. The pattern of epithelial and endothelial Ia^d expression was not homogeneous and the intensity of M5/114 staining correlated with the frequency of CD3+ T-lymphocytes in the adjacent epithelium, subepithelium, and lamina propria (Fig. 5*c*). Antibiotic treatment was associated with a dramatic decrease in the numbers of intravillous CD3-positive cells: after 3 weeks, practically all had disappeared from all parts of the villi (Fig. 5*d*) and associated lymphoid deposits, and there was a coincident decline in Ia^d expression on surrounding epithelial and endothelial cells. The majority of intravillous T-cells were CD8+ CD4- (data not shown).

Specific Localization of i.v. Administered Anti-Ia^d Antibody to Tumor Vasculature, B-Cells, and Macrophages in Antibiotic-treated Nude Mice. Tumor-bearing BALB/c *nu/nu* mice were given i.v. injections of anti-Ia^d or the isotype-matched control antibody and euthanized 1, 4, or 24 h later. The *in vivo* localization of anti-Ia^d antibody in tumor and normal tissues is shown in Table 1 and Fig. 6. Anti-Ia^d antibody was found on the luminal membrane and in the cytoplasm 6b) of most or all tumor vascular endothelial cells 1 h after injection (Fig. 6, *a* and *b*). A similar pattern was seen at 4 h after injection, but by 24 h the labeling of tumor endothelial cells was weaker and entirely intracellular, consistent with the progressive internalization and metabolism of the antibody by endothelial cells (Table 1). Also, at 24 h small amounts of antibody were detectable in the immediate perivascular regions of the tumor.

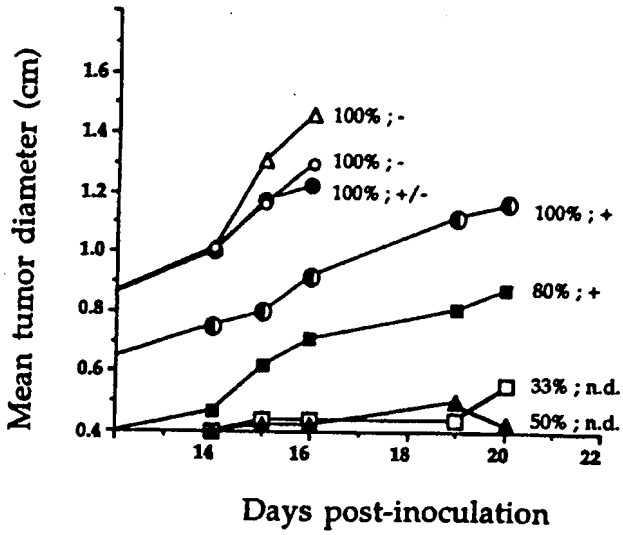

Fig. 3. Tumorigenicity, growth, and tumor endothelial cell Ia^d expression in pure and mixed s.c. C1300 and C1300(Muγ) tumors. BALB/c *nu/nu* mice were given injections of a total of 2×10^7 tumor cells in which the ratios of C1300:C1300(Muγ) cells were 10:0 (Δ), 9:1 (○), 8:2 (●), 7:3 (◐), 5:5 (■), 3:7 (▲), or 0:10 (□). Vertical axis, mean diameter of the tumors at various times after injection. Also shown are the percentage of animals in each group that developed tumors. The proportion of Ia^d-positive vascular endothelial cells was categorized as follows: +, 75–100%; +/−, 25–75%; −, 0–5%; n.d., not determined because no intact blood vessels were visible. SD were <15% of mean diameters and are not shown.

Table 1 *Localization of i.v. administered anti-Ia^d antibody in C1300(Muγ) tumor-bearing mice*[a]

Tissue	Antigen expression	Localization in vivo		
		1 h	4 h	24 h
Tumor[b]	EC,[c] Mϕ	EC[d]	EC	EC[e]
Brain	None	None	None	None
Colon[a]	Some crypt epithelium and EC, Mϕ	None	None	None
Ileum[a]	Some villous epithelium and EC, Mϕ	None	None	None
Heart	Interstitial Mϕ	None	None	None
Kidney	Occasional tubule epithelium, Mϕ	None	None	None
Liver	KC, numerous Mϕ in parenchyma	KC[d]	KC	KC,[e] some Mϕ
Lung	Numerous Mϕ in parenchyma	None	None	None
Pancreas	Numerous Mϕ in parenchyma	None	None	None
Skin[f]	Langerhans cells	None	None	None
Spleen	RP Mϕ, MZ Mϕ, MZ B cells, some lymphocytes in WP	MZ	MZ, WP	MZ, RP, WP
Bone marrow	5–8% of cells	ND	ND	<5% of cells

[a] Experiments performed with antibiotic-treated BALB/c *nu/nu* mice.
[b] Mixed tumor of 7:3 C1300:C1300(Muγ) cells grown s.c.
[c] EC, endothelial cells; KC, Kupffer cells; Mϕ, macrophages; MZ, marginal zone; RP, red pulp; WP, white pulp; ND, not determined.
[d] Strong staining, including discernable labeling of luminal membranes.
[e] Weaker staining, entirely intracellular.
[f] Either adjacent to, or distant from tumor.

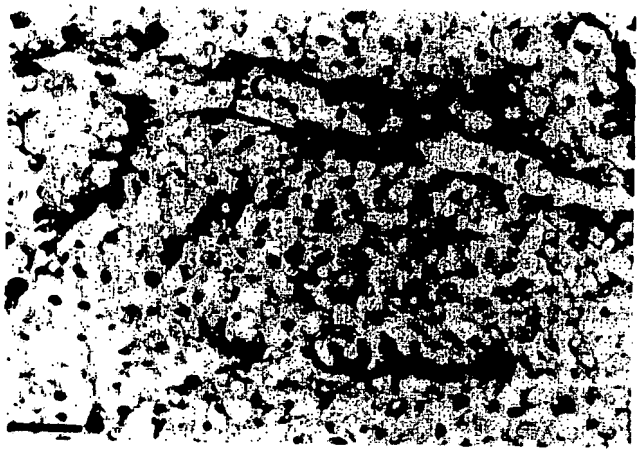

Fig. 4. Expression of Ia^d on vascular endothelial cells and macrophages in mixed C1300:C1300(Muγ) tumors. Frozen sections of the tumor were stained with anti-Ia^d antibody, M5/114, using the indirect immunoperoxidase technique. Capillary endothelial cells (EC) and interstitial macrophages (M) are intensely stained. *Bars*, 20 μM.

Anti-Ia^d antibody was bound to Kupffer cells in the intravascular compartment of the liver within 1 h of injection (Fig. 6*c*). At later times after injection, internalization and degradation of the antibody were apparent (Table 1). Adjacent sinusoidal endothelial cells were not stained (Fig. 6*c*). The high permeability of hepatic fenestrated endothelia was indicated by the penetrance of the antibody to reach some hepatic parenchymal macrophages (Table 1). In the spleen, perivascular B-cells and macrophages in white pulp marginal zones were stained within 1 h, showing that the vasculature of this organ was particularly permeable to antibody (Fig. 6*d*). At later stages, the antibody penetrated throughout the splenic lymphoid compartment and also labeled a minority of red pulp macrophages (Table 1). In organs other than the liver and spleen, macrophages and related cells such as the Langerhans cells of the skin were unstained probably because their vascular endothelium contains tight junctions and is relatively impermeable to antibodies. After 24 h, a small percentage (<5%) of bone marrow cells were labeled (Table 1).

Anti-Ia^d antibody was bound to some endothelial cells in the ileum, duodenum, and colon of BALB/c *nu/nu* mice, but not elsewhere in the intestine, 1 h after injection. Antibiotic treatment for 1–3 weeks before injection of anti-Ia^d antibody completely abolished localization to gut endothelial cells. The isotype-matched control antibody was not detected in tumor or normal tissues at any time after injection.

Taken together, these results strongly indicate that, when injected into appropriate tumor-bearing animals anti-Ia^d antibody or immunoconjugates will localize effectively to most or all tumor endothelial cells while sparing life-sustaining normal tissues.

Perivascular Staining of Tumor Cells in Mice Given Injections of Antitumor (H-2K^k) Antibody. When frozen sections of s.c. tumors deriving from inocula of mixed C1300 and C1300(Muγ) cells (7:3) were stained with biotinylated anti-H-2K^k antibody, a homogeneous staining pattern was obtained (Fig. 7*a*). The levels of IFN-γ secreted by the C1300(Muγ) cells in the tumor were therefore sufficient to induce increased H-2K^k expression by the C1300 component of the tumor, in accordance with the *in vitro* co-culture experiments described above. The staining was specific because no staining was seen with the isotype-matched control antibody. No specific labeling of any normal tissue by anti-H-2K^k antibody was found, as expected since this antibody was raised in an H-2^d mouse strain.

In contrast with the rapid binding of i.v.-administered anti-Ia^d antibody to tumor vasculature, no significant accumulation of anti-H-2K^k antibody was apparent 1 h after injection. After 4 h, however, anti-H-2K^k antibody was detected in small islands of tumor cells surrounding central capillaries (Fig. 7*b*). After 24 h, the antibody was bound to larger discrete areas of tumor cells, but staining intensity was diminished relative to the earlier time points. Even with localization times of up to 72 h, homogeneous labeling of all tumor cells was not achieved (data not shown).

No localization of anti-H-2K^k antibody was found in any normal tissues, and binding of the isotype-matched control antibody was not detectable in tumor or normal tissues.

DISCUSSION

This report describes a murine model for studying the antibody-directed targeting of vascular endothelial cells in solid tumors. In this model, IFN-γ gene-transfected tumor cells growing in antibiotic-treated nude mice release IFN-γ, which induces the *de novo* expression of MHC Class II antigens on the tumor vasculature. MHC Class II is absent from the vasculature in the normal tissues of these mice and hence the Class II induced on the tumor vascular endothelial cells serves as a specific marker. Class II is present on B-lymphocytes, Kupffer cells, and other cells of monocyte/macrophage lineage, but

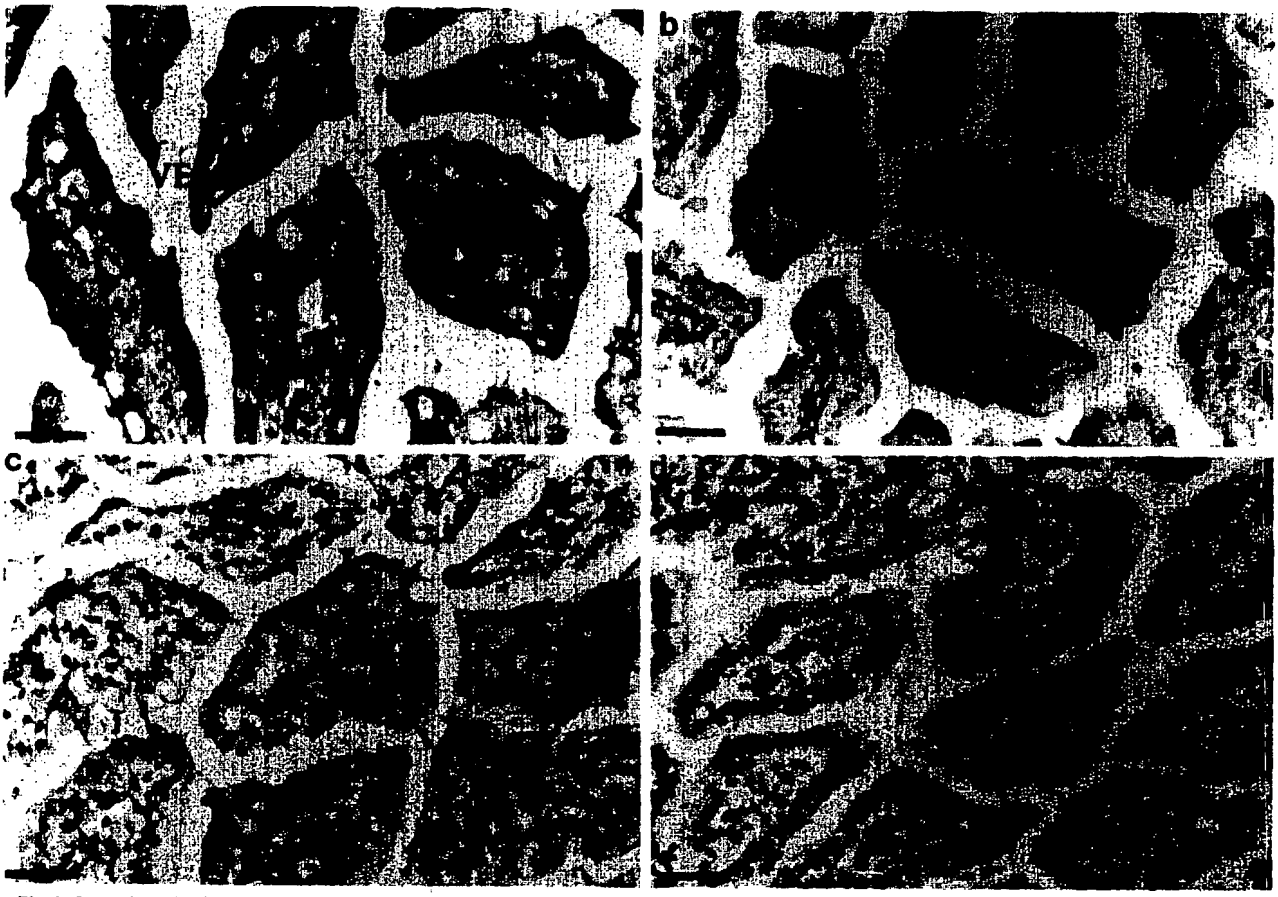

Fig. 5. Reduction of Ia^d expression and CD3+ cell infiltration in the colon of antibiotic-treated mice. Frozen sections of colon from untreated (*a* and *c*) and antibiotic-treated (*b* and *d*) BALB/c *nu/nu* mice were stained with anti-Ia^d antibody, M5/114 (*a* and *b*), or anti-CD3 antibody (*c* and *d*). In untreated mice (*a*), there is heavy labeling of Ia^d on intravillous capillaries (EC) and villous epithelium (VE), whereas in antibiotic-treated mice (*b*) there is light staining of Ia^d on some epithelial cells (VE) but capillaries (EC) are unstained. Similarly, in untreated mice (*c*) numerous small round CD3+ cells are visible in villous connective tissue (*arrowheads*), whereas in antibiotic-treated mice (*d*) only occasional epithelium and CD3+ cells are seen (*arrowheads*). *Bars*, 20 μM.

these cells are not life-sustaining so their temporary absence after targeting with cytotoxic immunoconjugates should be tolerable. IFN-γ also induces the tumor cells themselves to express high levels of the MHC Class I antigen, H-$2K^k$, which can serve as a tumor cell-specific marker in BALB/c *nu/nu* mice, which carry the H-$2K^d$ haplotype. Thus, anti-Ia^d and anti-H-$2K^k$ antibodies injected systemically localize selectively to tumor vascular endothelial cells and tumor cells, respectively, which enables the approaches of targeting the tumor vasculature and the tumor cells to be compared in this model, or used in combination.

It was necessary to dilute the C1300(Muγ) cells with C1300 parental cells in the ratio 3:7 to establish progressively growing s.c. tumors in which the vascular endothelial cells were Class II (Ia^d)-positive. Undiluted C1300(Muγ) cells were poorly tumorigenic in BALB/c *nu/nu* mice, in contrast with a prior report (30). Vascular dysfunction appeared to be the reason why pure C1300(Muγ) tumors would not grow beyond a diameter of 5–6 mm. Staining of sections of tumors with the anti-endothelial cell antibody MECA 20 revealed that the vessels were morphologically atypical with no visible lumens. It is possible that excessively high intratumoral IFN-γ levels in pure C1300(Muγ) tumors caused direct vascular toxicity or activated macrophages in the tumor to become cytotoxic for endothelial cells (44), although the number, location, and activation status of Mac-1+ macrophages and CZ-1+ NK cells did not vary significantly between C1300, C1300(Muγ), and mixed tumors.

Anti-Ia^d antibody injected i.v. bound rapidly and homogeneously to vascular endothelial cells in the tumor, confirming the immediate accessibility of intravascular targets (9). Remarkably, the inductive influence of IFN-γ from C1300(Muγ) cells was completely restricted to the tumor mass: endothelial cells in the overlying area of skin expressed no detectable Ia^d and did not bind any i.v.-injected anti-Ia^d antibody. It is likely that IFN-γ entering the systemic circulation is neutralized by a specific binding protein, perhaps a soluble form of the IFN-γ receptor (45), whose normal role may be to down-regulate cytokine activity (46) or to restrict it to the immediate locale of secretion.

Ia^d antigens are not restricted solely to tumor endothelial cells. MHC Class II antigens are expressed constitutively by B-cells, activated T-cells, and cells of the monocyte/macrophage lineage in humans and rodents (47, 48) and were found in this study also to be present on occasional proximal tubules in the kidney and on some epithelial cells in the intestine of antibiotic-treated BALB/c *nu/nu* mice. However, when injected i.v., only the hepatic Kupffer cells, splenic B-cells, and macrophages in the liver and spleen bound detectable amounts of the anti-Ia^d antibody: the potentially life-sustaining Class II-positive renal and gut epithelial cells were unstained. Localization of i.v.-injected anti-Ia^d antibody to hepatic Kupffer cells and splenic marginal zone B-cells occurred within 1 h, in accordance with the report of Kennel *et al.* (9). Presumably, the extreme permeability of the discontinuous splenic endothelium permits rapid

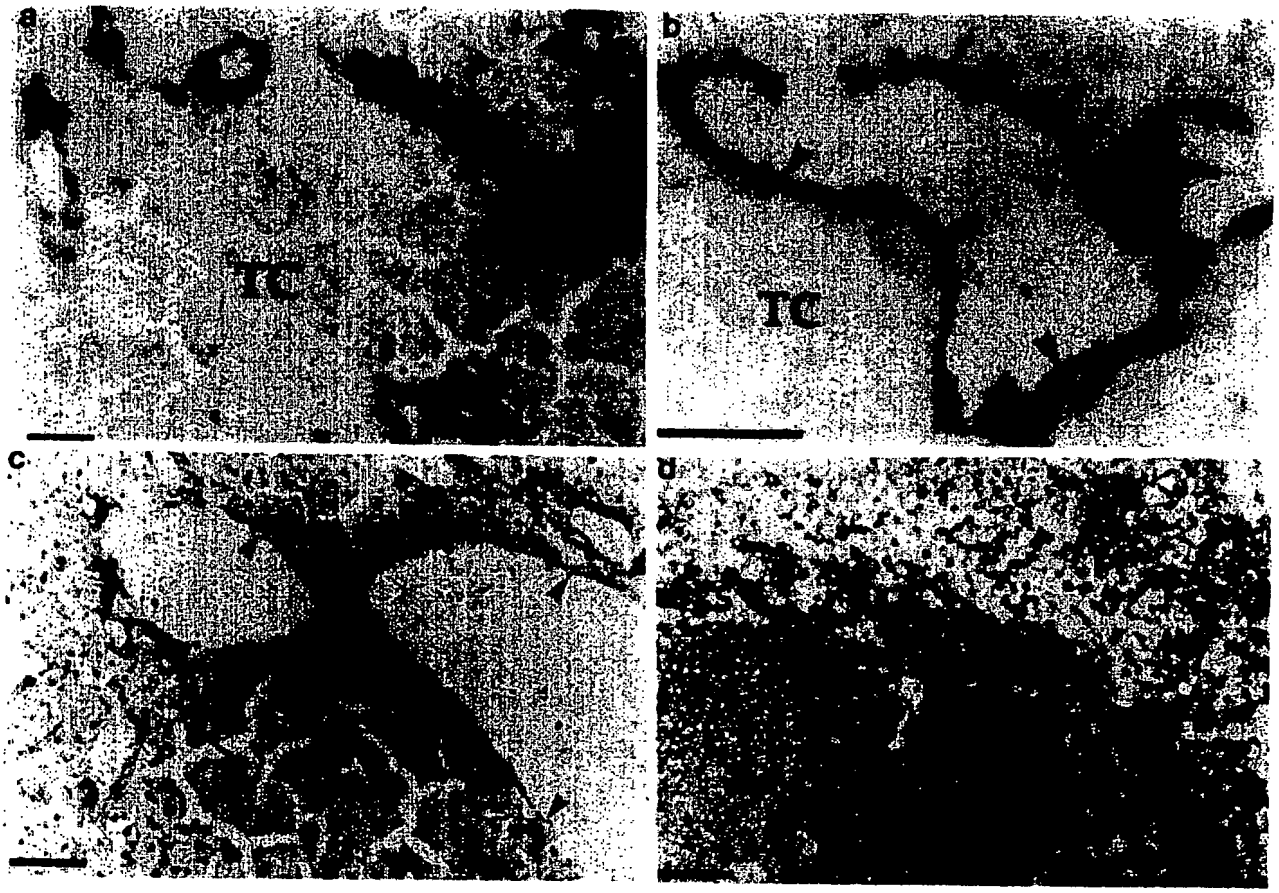

Fig. 6. Specific localization of i.v.-administered anti-Ia^d antibody in nude mice. Anti-Ia^d antibody, M5/114, was injected and 1 h later the tumor, liver, spleen, and colon were removed. Frozen sections were then stained for rat IgG using the indirect immunoperoxidase technique. In the tumor (*a* and *b*), all capillary endothelial cells (EC) are heavily labeled, but macrophages (M) and tumor cells (TC) are unlabeled. The localized anti-Ia^d antibody is particularly concentrated on the luminal membranes of tumor endothelial cells (*b, arrowheads*). *c*, in the liver, Kupffer cells (K) in sinusoidal crypts are stained but adjacent sinusoidal endothelial cells (*arrowheads*) are unstained. *d*, in the spleen, B-cells and macrophages adjacent to marginal zone sinusoids (S) are heavily labeled and there is some penetration of antibody deeper into the white pulp (WP). *Bars*, 10 μM (*a*), 20 μM (*b-d*).

extravasation of antibodies into the parenchyma of this organ and staining of the marginal zone B-cells (9). The reason for the lack of staining of renal and gut epithelial cells is probably that these cells are not readily accessible to i.v.-administered antibody because the antibody would have to diffuse across basement membranes and several tissue layers to reach these cells. In addition, it is likely that all the remaining anti-Ia^d antibody in the circulation was absorbed by more accessible splenic white pulp lymphocytes before significant extravasation into the red pulp (9, 11) or other normal tissues could occur. This is important because it illustrates a potentially critical pharmacokinetic difference between vascular targeting and tumor cell targeting. Because the tumor endothelial cells are so accessible to i.v.-administered antibody, the presence of a large "sink" of competing antigen in the blood or lymphoid organs should not prevent the antibody from reaching the target cells but should protect antigen-positive cells in most extravascular compartments. It is conceivable that an antibody recognizing a tumor vascular endothelial cell antigen that is shared by epithelial cells, for instance, might be targeted without the toxic side effects that have complicated therapy with anti-tumor cell immunoconjugates (49). Furthermore, even in the absence of such a sink, it is possible that operative specificity for tumor endothelial cells could be achieved in the face of cross-reactivity with extravascular normal tissues by decreasing the dose or by using rapidly cleared antibody fragments in the construction of the immunoconjugate. Anti-Ia^d antibody did localize to a small population of bone marrow cells, which were probably late stage myeloid progenitors. However, destruction of this population was not permanent or life threatening since no late occurring toxicity was seen in any animals and, 20 days after treatment with an anti-Ia^d immunotoxin, bone marrow aspirates contained unchanged numbers of granulocytes, monocytes/macrophages, and Ia^d+ cells.[4]

Although anti-Ia^d antibody did not localize to life-sustaining Ia^d+ extravascular tissues such as kidney tubules and gut epithelium, it did bind to gut endothelial cells in non-antibiotic-treated BALB/c *nu/nu* mice. These cells were as accessible as tumor endothelial cells and were required for survival since some regular BALB/c mice treated with high doses of M5/114 immunotoxins died from intestinal damage.[4] Murine endothelial cells do not express MHC Class II antigens *in vitro* (31, 50) or *in vivo* (51) unless stimulated with IFN-γ, so it is likely that induction of Ia^d on intestinal endothelial and epithelial cells was a result of local secretion of IFN-γ by T-cells (38) or activated NK cells (52, 53) in response to gut flora. In accordance with this view, numerous CD3+, CD8+ T-cells were observed in the villous stroma and epithelium of BALB/c *nu/nu* animals and their frequency correlated with the intensity of staining of

[4] F. J. Burrows, J. P. Overholser, and P. E. Thorpe, manuscript in preparation.

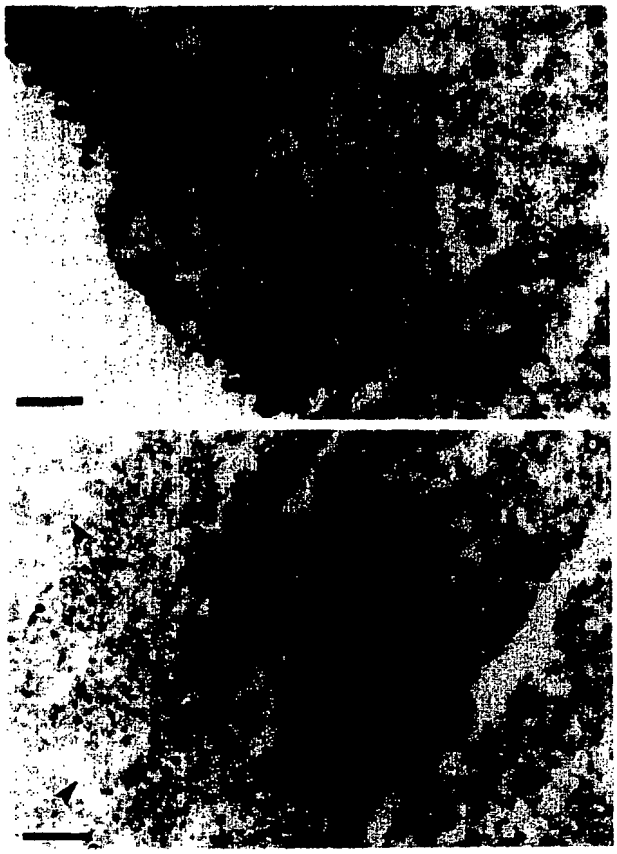

Fig. 7. Expression of H-2K^k and localization of i.v.-administered anti-H-2K^k antibody to perivascular regions in mixed C1300:C1300(Muγ) tumors. *a*, frozen sections of the tumor were stained with biotinylated anti-H-2K^k antibody, 11-4.1, using the streptavidin-biotin complex-horseradish peroxidase technique. Tumor cells show homogeneous staining by the anti-H-2K^k antibody. *Bars*, 20 μM. *b*, biotinylated anti-H-2K^k antibody was injected into a tumor-bearing BALB/c *nu/nu* mouse and 4 h later the tumor was removed. Frozen sections were stained with streptavidin-biotin complex-horseradish peroxidase. Tumor cells in the center of the field are heavily stained but others appear to have bound little or no antibody (*arrowheads*). *Bars*, 20 μM.

endothelial and epithelial cells with anti-Ia^d antibody. Furthermore, p.o. administration of tetracycline-HCl (a broad spectrum antibiotic) reversed T-cell infiltration, diminished Ia^d expression, and abolished localization of i.v.-injected anti-Ia^d antibody to gut endothelial cells. Antibiotic treatment had no effect on Ia^d expression by tumor endothelial cells. Most of the intravillous CD3+ cells observed in regular BALB/c *nu/nu* ileum were located within or immediately beneath the epithelial cell layer and had the phenotype CD4-CD8+, so it is most likely that they were thymus-independent T-cell receptor-$\gamma\delta$+ intraepithelial lymphocytes, which are present in BALB/c *nu/nu* mice (54), migrate in response to normal gut microbial colonization (54), and secrete IFN-γ (55).

Consistent with the findings of others (7, 9, 56, 57), an antitumor antibody directed against the H-2K^k antigen on C1300 and C1300(Muγ) cells showed perivascular staining of tumor cells after i.v. administration. In view of the homogeneous expression of H-2K^k by tumor cells *in vitro* and in sections of s.c. tumors, it is likely that the uneven intratumoral distribution of i.v.-injected anti-H-2K^k antibody was related to the vascular and interstitial physiology of the tumor (8, 11). This nicely demonstrates, in a single system, the limitations of using antitumor antibodies for targeting and the virtues of tumor vascular targeting. It may be possible to combine both approaches to advantage because the tumor cells that survive destruction of intratumoral blood vessels are likely to be those at the periphery of the tumor mass, close to the tumor-host interface. These areas are likely to be well vascularized by capillaries in adjacent normal tissues and have low interstitial pressure (8), so the surviving cells should be amenable to attack by antitumor immunoconjugates.

In summary, we describe a murine model with which to test the feasibility of targeting the vasculature of solid tumors. The model permits the antitumor effects of immunoconjugates directed against tumor vasculature to be compared with those of immunoconjugates directed against the tumor cells themselves. A forthcoming report will describe the effects of immunotoxins on large solid tumors in this model system.

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